Novel stromal-derived oncogenic signals enhance Ras-mediated cell proliferation

Undergraduate Honors Thesis

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Markus E. Harrigan

Undergraduate Biomedical Science Major
School of Health and Rehabilitation Sciences
The Ohio State University

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Thesis Committee:
Dr. Gustavo W. Leone, Ph.D., Advisor
Dr. Adriana Dawes, Ph.D.
Dr. Ajit Chaudhari, Ph.D.

ABSTRACT

The appreciated, yet relatively unexplored, role of the complex tumor microenvironment in cancer progression provides a novel avenue to target cancer. Oncogenic signaling networks between stromal and cancer cells inherently exist, but have yet to be readily identified, and more importantly, understood at the molecular level. To systematically identify these signaling networks, we utilized the well-characterized vulvagenesis program of *Caenorhabditis elegans* (*C.* elegans). During vulva development, mesenchymal cells signal to adjacent epithelial vulva precursor cells (VPCs) through the Ras signaling pathway to promote the cell proliferation and patterning that form a mature vulva. This developmental signaling is akin to the cellular signaling interactions between stromal mesenchymal cells and epithelial cancer cells within a tumor. Consistent with their hallmark role in the formation of many human cancers, activating mutations in the RAS (*let-60*) oncogene in *C. elegans* lead to the hyper proliferation of epithelial VPCs, which presents as a multiple-vulva (MUV) phenotype. To elucidate signaling networks derived from the mesenchymal cells (which model stroma) that promote hyper proliferation in epithelial VPCs (which model cancer cells) in the context of mutant RAS (*let-60*), we conducted a genome-wide “stroma-specific” RNAi screen in *C. elegans*. The screen identified 60 “candidate genes”, 42 with corresponding mammalian orthologs, whose activity in the mesenchymal cells contribute to the epithelial MUV phenotype. Subsequent studies were initiated to probe the mechanisms and pathways through which these “candidate genes” act. The overarching

challenge is to translate the identified mammalian orthologs into clinically relevant findings that target the tumor microenvironment of Ras-driven cancers.

ACKNOWLEDGEMENTS

I would first like to thank and extend my appreciation to Dr. Gustavo Leone for his guidance and support throughout my undergraduate research career. Under his guidance I quickly became fascinated with the experimental process and its potential to solve the complex puzzles that nature and disease present. Dr. Leone's encouragement to approach problems from exciting unconventional angles is what motivated me to pursue a career in science. I am extremely thankful for the time and thought that Dr. Leone has invested in my undergraduate research career, as well as this project.

I would also like to thank Dr. Helen Chamberlin as well as previous and current members of the Leone Lab including Dr. Nicholas Sullivan, Dr. James Dowdle, Komal Rambani, Huayang Liu, Markus Mair, Piotr Daniel, Raleigh Kladney, Kyle Toth, Connor Sullivan, Serena Chang, Michael Lause, and Karl Eiring. Every lab member has played an important role, in one way or another, in the learning process I experienced over the past three and a half years.

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Finally, I would like to thank my family, especially my parents Christopher Harrigan and Heidi Beke-Harrigan, and friends for their unwavering support. I would not be where I am today, nor the person who I am today, if it were not for them.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1 The tumor microenvironment as a facilitator of tumor progression:

Solid tumors are comprised of cancerous cells as well as a heterogeneous environment of fibroblasts, extracellular matrix, endothelial cells, immune cells and other stromal components [1]. Tumor stroma and wound-healing stroma are both compromised of a myriad of fibroblasts, a striking histological similarity that may highlight the important role of fibroblasts in the tumor microenvironment [2]. In addition to visual similarity, tumor stromal fibroblasts function in an analogous manner. They stimulate ECM growth and release cytokines and growth factors through intercellular signaling in an attempt to “heal” the prevailing carcinoma *in situ* [3]. Notably, wound-healing fibroblasts are deactivated following their restorative function while tumor-stromal fibroblasts remain activated. Consequently, the constant signaling between stromal-fibroblasts and cancer cells is believed to result in cancer progression and development [4]. Our lab has confirmed the role of *PTEN* in stromal-fibroblasts in cancer development. *PTEN* was knocked-down in stromal fibroblasts in mouse models in which the oncogene *ErbB2* (*HER2*) was overexpressed in the mammary epithelial cells through the MMTV promoter. The consequence was an increase in tumor incidence, tumor size, and carcinoma progression, highlighting the key role of stromal-fibroblasts in reprogramming gene expression in the mammary-gland

microenvironment (Figure 1) [5]. This was the first instance that our lab identified the *in vivo* influence of stromal genes on tumor development.

1.2 The KRAS-mutant tumor microenvironment:

The KRAS signaling pathway, a growth factor propagator, plays a vital role in one of the deadliest human cancers, pancreatic adenocarcinoma (5 year survival rate: 5.8%), since over 95% of cases involve a mutation in the *KRAS* gene [6][7]. Furthermore, nearly 40,000 American lives will be lost to pancreatic adenocarcinoma in 2015, making it the 4th deadliest type of cancer in the U.S. [8]. Hyperactive KRAS signaling is also associated with lung and colorectal cancers while the HRAS and NRAS signaling pathways, completing the RAS family, are also well characterized human cancer provokers. Dense tumor stroma proliferation, known as desmoplasia, is a hallmark of pancreatic cancer created by the abundance of pancreatic fibroblasts and human pancreatic stellate cells (HPSC), cells that function analogously to fibroblasts [9][10][11]. Conditioned medium containing HPSCs, isolated from pancreatic adenocarcinoma, triggered increased in vitro tumor cell proliferation, migration, invasion, and resistance to radiation and gemcitabine in pancreatic cancer cell lines. Injection of HPSCs and pancreatic tumor cells in an orthotropic model produced equivalent results: increased tumor size and metastasis [9]. Additionally, irradiated stromal-fibroblasts increase the invasiveness of pancreatic cancer, rendering radiation treatment counterproductive [12]. Pancreatic cancer treatment is further undermined through tumor-stroma signaling that establishes chemoresistance [13]. Since effective treatments for pancreatic adenocarcinoma are **nonexistent**,

novel therapeutic strategies targeting stromal-fibroblast derived cell non-autonomous signaling networks that interact with the KRAS pathway to facilitate pancreatic tumor progression may be a useful therapeutic intervention.

1.3 Modeling the *KRAS*-mutant tumor microenvironment in *C. elegans* vulva development

Caenorhabditis elegans (C. elegans) are a very small, about 1mm in length, nematode that feed on bacteria, such as *Escherichia coli* (*E. coli*), and are easily cultivated and housed. Additionally, their well conserved genome, about 47% homologous with the human genome, paired with their short life and reproductive cycle, relative to mice, drastically expedites the experimental cycle, making *C. elegans* amenable to genome-wide/large-scale studies [14]. *C. elegans* vulva development is a tightly regulated process of cell division and migration that results in an organized 22-cell vulva. Importantly, this process depends on signaling between mesenchymal and epithelial cells while utilizing three highly conserved human cancer related pathways: Ras, Wnt, and Notch [15]. This signaling between mesenchymal and epithelial cells to promote the cell proliferation and patterning that leads to the formation of a mature vulva (the egg laying organ) is akin to the signaling interactions between a tumor and its microenvironment. In wild type *C. elegans* vulva development, three of the six vulva precursor cells (VPCs) are directed by the aforementioned signaling pathways (Ras, Wnt, Notch) to differentiate into mature vulva cells while the remaining three cells differentiate into non-specific hypodermal cells [16].

Contrary to the three *RAS* family genes in humans, the *C. elegans*

genome only houses one RAS gene (*let-60*). As a result, the *C. elegans* Ras signaling pathway corresponds to and is highly conserved with the KRAS signaling pathway as well as the HRAS and NRAS signaling pathways. Correspondingly, any findings in *C. elegans* could be applicable to KRAS, HRAS and NRAS related cancers. Specifically, the *let-60* gene is a member of the developmental Ras pathway, and furthermore, an ortholog of the human RAS proto-oncogene [17]. A gain-of-function (gf) *let-60* mutation in *C. elegans* hyper-activates the Ras signaling pathway, altering normal vulva development by influencing more than three VPCs to differentiate into mature vulva cells and ultimately resulting in the multiple vulva (MUV) phenotype. Although MUV is a much more controlled increase in proliferation than cancer, it still represents an undeniable increase in abnormal growth inducing signaling.

1.4 Hypothesis and Specific Aims

Cancer research has largely ignored the tumor microenvironment and focused almost exclusively on the role of cancer cells themselves. This leaves us with limited knowledge concerning the mechanisms and role of genetic signaling pathways in the entire tumor. Our main hypothesis is that the tumor microenvironment plays a crucial role in tumor growth and progression and, more specifically, that unidentified stromal genes exacerbate human tumor development in an oncogenic manner.

Specific aim 1: Identify novel stromal-derived oncogenic-like signals that provoke excessive RAS-mutant epithelial cell proliferation.

Mammalian models that study the tumor microenvironment are restricted to the analysis of one gene at a time. We overcame this limitation by conducting a genome-wide stroma-specific RNAi screen in *C. elegans* to systematically identify novel stromal-derived oncogenic-like signals that enhance Ras mediated cell proliferation.

Specific aim 2: *Functional analysis of "candidate genes" in the Ras signaling pathway.*

To partially address how identified "candidate genes" might be eliciting their oncogenic-like effect on epithelial cell growth I chose to use fluorescent reporters of the RAS signaling pathway (*egl17::CFP*) to visualize the activity of candidate genes relative to baseline RAS signaling in VPCs. Since the *let-60* mutation shows over-induction of RAS signaling compared to wild-type *let-60* (data not shown), RNAi knockdown of "candidate genes" that function through the Ras signaling pathway should diminish overall Ras reporter signaling while knockdown of genes that function downstream or in alternate pathways will not alter Ras reporter signaling [18]. The working hypothesis of this aim is that while some candidates will work through the Ras signaling pathway, others may be eliciting their oncogenic effect through alternative cross talking signaling pathways.

CHAPTER 2

MATERIALS AND METHODS

2.1 *C. elegans* strains:

C. elegans strains utilized in experiments include the 7.10, GS3582, and MT2124 strains. 7.10 was the strain utilized in the genome-wide screen and its properties include a *let-60* (Ras) gain of function mutation, *rrf-3* loss of function mutation, *rde-1* loss of function mutation, and gonadal, muscle, and anchor cell tissue-specific promoters of *rde-1*. The GS3582 strain expresses a fluorescent reporter construct (*egl-17p::CFP*) for the RAS signaling pathway while the MT2124 strain harbors a single *let-60* (Ras) mutation. All strains were developed in the lab of Dr. Helen Chamberlin and maintained in an incubator at 20 °C.

2.2 Tissue-specific RNAi construct in *C. elegans*

Our lab previously constructed and validated a “stroma”-specific RNAi model of *C. elegans*. To achieve a “stroma”-specific RNAi effect, RNAi machinery was inactivated via a mutation of the *rde-1* gene, which encodes a protein that is vital for transforming precursor RNA into functional dsRNA. In the *rde-1* mutant *C. elegans* strain, wild-type *rde-1* was reintroduced under the control of three stromal-specific promoters (anchor cell, somatic gonad, muscle) to achieve tissue-specific knock down. First, we demonstrated tissue-specific expression of GFP driven by stroma-specific promoters (Figure 2).

Functional validation of the stroma-specific RNAi model utilized *lin-3*/EGF and *lin-39*, which are both necessary in a tissue specific manner for normal vulva

development. The *lin-3* gene is required in “stromal” cells while *lin-39* function is required in VPCs for proper vulva formation [19][20]. Specifically, *lin-3* codes for a ligand (EGF ligand) released by “stromal” cells while *lin-39* codes for a receptor (EGFR) on epithelial VPCs that binds the aforementioned ligand. As expected, when either *lin-3* or *lin-39* were knocked down via RNAi in wild type *rde-1 C. elegans*, a vulvaless phenotype was observed (Figure 3A). Conversely, when either gene was knocked down in *rde-1* mutant *C. elegans*, a normal phenotype was observed (Figure 3B), as RNAi is not functional in this strain of *C. elegans*. Importantly, however, *lin-3* RNAi in “stroma specific” *rde-1 C. elegans* yielded a vulvaless phenotype (Figure 3C) while *lin-39* RNAi in “stroma-specific” r*de-1 C. elegans* produced a normal vulva phenotype (Figure 3D). These converse results illustrate the stroma-specific RNAi functionality of our model.

2.3 *C. elegans* RNAi screen and microscopy

To achieve stroma-specific knockdown, we crossed the aforementioned strain of “stroma-specific” *rde-1 C. elegans* with a *let-60* mutant strain. To increase *C. elegans’* sensitivity to RNAi, a loss-of function mutation in the *rrf-3* gene was also introduced, yielding our final 7.10 strain [21]. We systematically screened our strain against the Ahringer Laboratory *E. coli* RNAi library (Bioscience LifeSciences) targeting 16,757 *C. elegans* genes (81% of the *C. elegans* genome) [22]. RNAi was achieved by feeding the 7.10 strain individual *E. coli* clones expressing double stranded RNA (dsRNA) on a gene-by-gene basis. A lab technician was responsible for inoculating *E. coli* RNAi clones from 384-well library plates into 96-well deep-well plates (VWR) with Luria broth (LB)

medium (Invitrogen) containing 50 µg/mL carbenicillin (Sigma-Aldrich) for 16 hours at 37°C. The cultured *E. coli* was then seeded onto 12-well plates (Corning) with standard nematode growth medium (NGM) agar, but containing double the amount of normal peptone, as well as 25 µg/mL carbenicillin and 1mM IPTG (Lab Scientific) and incubated at room temperature for 24 hours. Approximately 100 synchronized 7.10 embryos were seeded into each well of the 12-well plates and incubated for 4 days at 20°C with the cultured *E. coli*. For the primary genome-wide RNAi screen approximately 50 adult *C. elegans* in each well were phenotypically scored for MUV and wild type vulva phenotypes using an Olympus SZ60 dissection microscope. 90% of *C. elegans* in the 7.10 strain express the MUV phenotype due to hyperactive Ras signaling (*let-60* mutation). Correspondingly, genes that yielded a significant reduction in the MUV phenotype (>40% reduction) of a *C. elegans* population when knocked down by *E. coli* clones were evaluated twice more in secondary and tertiary screens. Ultimately, genes that yielded significant reductions in the MUV phenotype upon stromal-specific RNAi in at least two out of three replications were designated as primary “candidate genes”. For practical reasons, the primary, secondary, and tertiary screens were carried out with 2 other lab members, James Dowdle, Ph.D., and Komal Rambani, M.S. I personally screened 4,226 genes in the primary screen (25.2% of the genome) and 110 genes in both the secondary and tertiary screens. Primary “candidate genes” were individually re-evaluated, also using the 12-well plate RNAi platform, but in this instance plates were incubated at 20°C for 48 hours and only L4 stage worms were selected for imaging. DIC

images were captured with a Spot RT Monochrome digital camera (Diagnostic Instruments) using a Zeiss Axioskop2 microscope to confirm the reversion of the MUV phenotype to wild type vulva phenotype upon stromal “candidate gene” depletion at high magnification (1000x). Komal Rambani conducted a majority of these high-magnification experiments, but I contributed to the experiments evaluating the “candidate genes” from chromosomes III and IV.

2.4 Generation of male *C. elegans*

GS3582 males were generated by “heat shock treatment”: the incubation of 5 hermaphrodite *C. elegans* at the L4 larval stage for exactly 6 hours at 30°C [23]. This treatment yielded a small population of males, which were subsequently mated with sibling hermaphrodites to generate a larger population of male *C. elegans*.

2.5 Ras reporter genetic cross

GS3582 males were crossed with 7.10 hermaphrodites (Figure 4). The fluorescent reporter construct (*egl-17p::CFP*) in the GS3582 strain exhibits an easily identifiable GFP marker in the neural region of *C. elegans* while the 7.10 strain expresses different visual markers: MUV phenotype (as a result of the *let-60* mutation), and a global GFP expression pattern (as a result of the tissue-specific *rde-1* promoters). The *rrf-3* and *rde-1* mutations in the 7.10 strain express no visually traceable markers.

To initiate the cross, three GS3582 males at the L4 larval stage were plated with five 7.10 hermaphrodites at the L4 larval stage and incubated at 20°C.

5 days later, 25 F_1 progeny displaying the three visual markers (neural GFP, multiple vulva phenotype, global GFP) were isolated and allowed to self-propagate. Six F_2 progeny were selected and isolated based on the same visual markers and subsequently allowed to self-propagate to confirm true-breeding of the markers, and the corresponding homozygous presence of the fluorescent reporter, *let-60* mutation, and *rde-1* tissue-specific promoters. In total, two independent crosses were initiated and executed to completion.

2.6 *C. elegans* DNA extraction:

C. elegans populations were expanded until near-starvation, harvested, and mixed with 148.2 µL worm-lysis buffer and 1.8 µL proteinase K (5 mg/mL). The mixture was stored overnight at -80 °C to enhance cuticle cracking. A PCR machine was then utilized to heat the mixture to 65 °C for 1 hour, followed by 95°C for 15 minutes. The resulting genomic DNA (gDNA) was stored at 4°C and used for all subsequent PCR experiments.

2.7 Genotyping - PCR and electrophoresis:

Since the *rrf-3* and *rde-1* mutations display no phenotypic markers, their presence was probed through PCR and, in the instance of the *rrf-3 mutation*, the subsequent analysis of DNA amplification products via electrophoresis. *rrf-3* was amplified by 20 µL PCRs (Table 1) and run out on a 2% agarose gel at 120 V for 35 minutes and analyzed with a FluorChem E System imager (ProteinSimple). *rde-1* was amplified via 50 µL PCRs (Table 2). The *rde-1* mutation is a point mutation, resulting in the inability to detect a difference between the WT and

mutant gene amplification via electrophoresis. Accordingly, 10 µL of each *rde-1* amplifying PCR was analyzed by electrophoresis simply to confirm the presence of amplified *rde-1*.

2.8 Genotyping - sequencing:

The remaining 40 µL from *rde-1* amplifying PCRs was purified with a PCR Purification Kit (Qiagen) to obtain pure DNA. The concentration of isolated DNA was measured with a spectrophotometer (NanoDrop, Wilmington, Delaware, USA) and then submitted for sequencing at The Ohio State University sequencing core facility.

CHAPTER 3

RESULTS

3.1 Stromal factors contribute to Ras-mediated epithelial cell proliferation

The Ahringer Laboratory *E. coli* RNAi library containing 16,757 RNAi clones, which accounts for approximately 81% of the *C. elegans* genome, was used to systematically feed *stroma-rde-1;let-60(-);rrf-3(-)* larvae RNAi inducing *E. coli* clones on a gene-by-gene basis [22]. A >40% reduction in MUV phenotypes was used as the primary end-point for reduced abnormal Ras-driven VPC proliferation. RNAi that resulted in significantly reduced vulva defects, MUV, in at least two of three independent replicates were classified as “candidate genes”. Ultimately, screening over 16,000 genes with stroma-specific RNAi identified 60 stromal “candidate genes” that contribute to the hyper proliferation of epithelial vulva cells in the context of hyperactive Ras signaling.

Amino acid sequence alignment to the mouse and human proteome revealed that 42 of the 60 (70%) final “candidate genes” correspond to 39 mammalian orthologs (Table 3) (*his-73, his-59, and his-32* share the ortholog HIST2H3D while *his-43* and *his-3* share the ortholog HIST2H2AB), whereas only 47% of all genes in the RNAi library have a mammalian ortholog. This increased level of conservation might suggest that stroma-tumor signaling networks revealed by this screen are conserved between *C. elegans* and humans. Interestingly, many candidate genes can also be grouped into specific processes, such as: histones, membrane proteins (transporters and receptors), ribosomal

components, metabolism, and cell cycle/DNA regulators. Although more detailed computational analysis is required, the striking convergence of stromal hits on a few selected processes seems promising. Additionally, high magnification (1000x) validation of candidate genes from chromosomes III and IV confirmed the vulva phenotypes observed at low magnification (Table 4).

3.2 Failed Ras reporter genetic cross

Two attempted crosses failed to generate a *C. elegans* strain with the desired genetic background: *let-60*(gf) mutation, *rrf-3*(lf) mutation, *rde-1*(lf) mutation, Ras Reporter construct (*egl17::CFP),* and gonadal, muscle, and anchor cell tissue-specific promoters of *rde-1*. In cross 1, out of 6 F_2 generation strains, all selected for visual markers confirming the presence of the *let-60* mutation, Ras Reporter construct, and tissue-specific *rde-1* promoters, strain 6 exhibited an *rrf-3* and *rde-1* mutant genotype (Table 5). Surprisingly, this genotype reverted to an *rrf-3* mutant and *rde*-1 heterozygous mutant genotype in the F_3 generation, and eventually an *rrf-3* mutant and *rde*-1 wild type genotype in in the F_4 generation (Figure 5). *rrf-3* PCR/electrophoresis genotyping (Figure 6) and *rde-1* sequencing (Table 6) of the F_4 generation of strain 6 represent the endpoint of cross 1. In cross 2, the F_2 generation of strain 10 displayed an *rrf-3* mutant and *rde*-1 heterozygous mutation (Table 7). However, similar to the occurrence in strain 6, strain 10 reverted to an *rrf-3* mutant and *rde*-1 wild type genotype in the F_4 generation (Figure 7).

In addition to following strains 1-12 to conclusion, 20 additional strains from crosses 1 and 2 were selected and followed. These strains generally carried

homozygous copies of 3 desired characteristics, 1 heterozygous characteristic, and heterozygous *rde-1* (for example: a strain that was homozygous for *rrf-3*(lf), Ras Reporter construct, tissue specific *rde-1* promoters, and heterozygous for *let-60,* and *rde-1*) in the F_2 generation. Similarly to strains 6 and 10, these strains either developed a wild type *rde-1* genotype or perished in later generations.

CHAPTER 4

DISCUSSION AND CONCLUSION

We hypothesized that unidentified stromal genes exacerbate human tumors in an oncogenic manner. Furthermore, we hypothesized that "candidate genes" identified through our screen would work through the Ras signaling pathway, while others could be eliciting their oncogenic-like effect through alternative cross-talking signaling pathways. In summary, there were two aims to this study:

- Identify novel stromal-derived oncogenic signals that enhance Ras-mediated cell proliferation.
- Evaluate the mechanism by which identified stroma-specific "candidate genes" promote excessive epithelial cell proliferation.

4.1 Novel stromal-derived oncogenic signals enhance Ras-mediated cell proliferation

While the Ahringer Laboratory *E. coli* RNAi library only targets 81% of the C. elegans genome, a more complete *E. coli* RNAi library does not currently exist so it was not possible for us to screen the remaining 14% of the *C. elegans* genome. Additionally, while only 47% of the C. elegans genome is conserved with the human genome our "candidate genes" showed a much higher level of conservation (70%). Regardless, not all identified "candidate genes" can progress to human validation experiments. Although these constraints are impossible to overcome, our systemic method is the first *in vivo* screen to identify

stromal oncogenic-like signaling networks that could be highly conserved in the tumor microenvironment.

As outlined previously, the contribution of the tumor microenvironment to tumor development and progression is evident, yet the detrimental mechanisms remain unclear, especially in regards to pancreatic cancer. Pancreatic cancer remains one of the deadliest cancers and evidence suggests that the few existing treatments actually contribute to tumor progression. Accordingly, time is of the essence when it comes to developing novel treatments for current and future pancreatic cancer patients. Exploiting the vulvagenesis program of *C. elegans* in a systematic manner to expose stromal signaling cascades that enhance Ras-mediated cell proliferation is the first step to elucidating the genetic contributions of the pancreatic tumor microenvironment. Additionally, our model proves more physiologically relevant than a potential static *in vitro* model in which the same stromal cells would lie adjacent to the same cancer cells through the duration of the experiment, as well as more financially feasible and time conscious than a genome-wide screen in a higher-level organism such as a mouse. Ultimately, we propose that the identified oncogenic-like stromal signaling pathways may provide the opportunity to develop exciting therapeutics that target novel stromal pathways in combination with traditional tumor cell targeting treatments.

4.2 Inability to establish a *stroma-rde-1;let-60(lf);rrf-3(lf);Ras Reporter C. elegans* strain

Based on the results of our cross, the derivation of our desired Ras Reporter model via a similar crossing strategy seems unlikely. Specifically, the

elusiveness of homozygous mutant *rde-1* in our cross suggests lethality or a possible growth disadvantage in our desired *C. elegans* strain.

4.3 Future Studies and Conclusions

An alternate approach to deriving a viable Ras reporter strain would be to mate male 7.10 *C. elegans* with hermaphrodite GS3582 *C. elegans*. Yet another approach would be to search the literature for the availability of a *C. elegans* strain harboring a reporter for a different Ras-targeted promoter. Perhaps crosses utilizing a reporter different from the GS3582 reporter would prove more successful.

If neither of the aforementioned strategies yield the desired strain then we could also construct a genetically simpler Ras reporter model. A *C. elegans* model consisting of just the Ras Reporter construct and a *let-60* mutation would not be as ideal, but would also display the effect of “candidate gene” knockdown on vulval Ras signaling. Such a model has been used in the past in our lab to evaluate “candidate genes” in a previous genome-wide screen.

In regards to the genome-wide screen, the possibility exists that our primary screen might have elicited false negative results. To combat this, we plan on grouping identified candidate genes by gene families. If a significant number of candidate genes are housed within a specific gene family then we can set up additional RNAi experiments for the genes in that family that were not identified as “candidate genes” in the initial genome-wide screen.

The possibility also exists that our stroma-specific RNAi model is not 100% stroma-specific. While we intended to only knockdown genes in the

mesenchymal cells in our experiments, it's possible that the RNAi effect "leaked" into the epithelial VPCs. A graduate student has developed a VPC-specific experimental strain to address this possbility. They are currently knocking down "candidate genes" in the VPC-specific strain and documenting the vulva phenotypes. If knockdown of the "candidate genes" in the VPC-specific strain does not significantly alter the 90% MUV phenotype, then our stroma-specific RNAi model is truly stroma-specific. However if the knockdown of "candidate genes" in the VPC-specific strain also reduces the MUV phenotype, then the possibility exists that our stroma-specific RNAi model is not truly stroma-specific and/or that the "candidate genes" are expressed in both the mesenchymal (stromal) and epithelial cells and that gene knockdown in either cell type is significant.

Identified "candidate genes" will be prioritized for further validation based on their expression in human pancreatic cancer associated fibroblasts (CAFs), which will be determined via RT-PCR. Candidate genes with the highest expression in pancreatic CAFs, relative to normal pancreatic fibroblasts, will be given priority. Candidate genes will first be validated by 2D, and subsequently by more physiologically relevant 3D, co-culture experiments that examine the effect of "candidate gene" knockdown in human pancreatic fibroblasts on human pancreatic epithelial cells. In order to produce an even more physiologically relevant tumor microenvironment model we also intend to conduct orthotopic murine model experiments that explore the influence of "candidate gene" knockdowns in human pancreatic fibroblasts on human pancreatic epithelial

cells. The aforementioned experiments will begin to explore whether stromal "candidate genes" identified in *C. elegans* may contribute to human pancreatic tumor development.

We plan to utilize fibroblasts as the primary stromal cell in our validation experiments because of their mesenchymal cell classification and abundance in the pancreatic tumor microenvironment. The mesenchymal characterization of fibroblasts is vital since in our developmental vulva model mesenchymal cells (anchor cell, gonad, muscle) signal to epithelial VPCs, similar to mesenchymal fibroblasts signaling to epithelial cancer cells in the pancreatic tumor microenvironment. If feasible, we are also interested in evaluating candidate genes in other stromal cell types, such as macrophages.

In conclusion, we identified 60 novel stromal factors, 42 with corresponding mammalian orthologs, that enhance Ras-mediated epithelial cell proliferation in *C. elegans* vulva development. Once confirmed as agitators of tumor growth and development in mouse and human models, these "candidate genes" could provide novel stromal specific drug targets and biomarkers for pancreatic cancer and other Ras-driven cancers.

LIST OF FIGURES

LIST OF TABLES

FIGURES

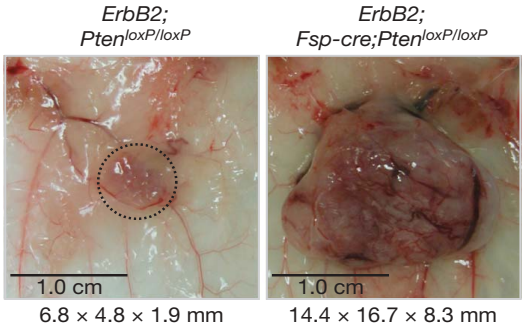

Figure 1. Before (left) and after (right) ablation of the *PTEN* gene in stromal fibroblasts surrounding an epithelial tumor harboring an *ErbB2* mutation.

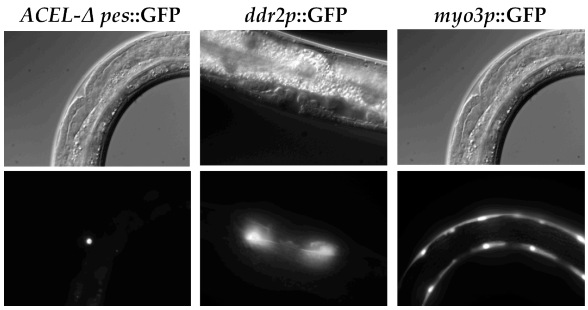

Figure 2. Stroma-specific promoters. Images courtesy of Huayang Liu.

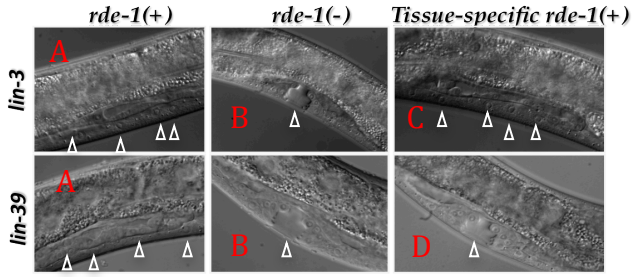

Figure 3. Stroma-specific RNAi confirmation. Please note that singular arrows point to mature vulvas while multiple arrows point to cells that should have become part of a mature vulva and thus represent a vulvaless phenotype. Images courtesy of Huayang Liu.

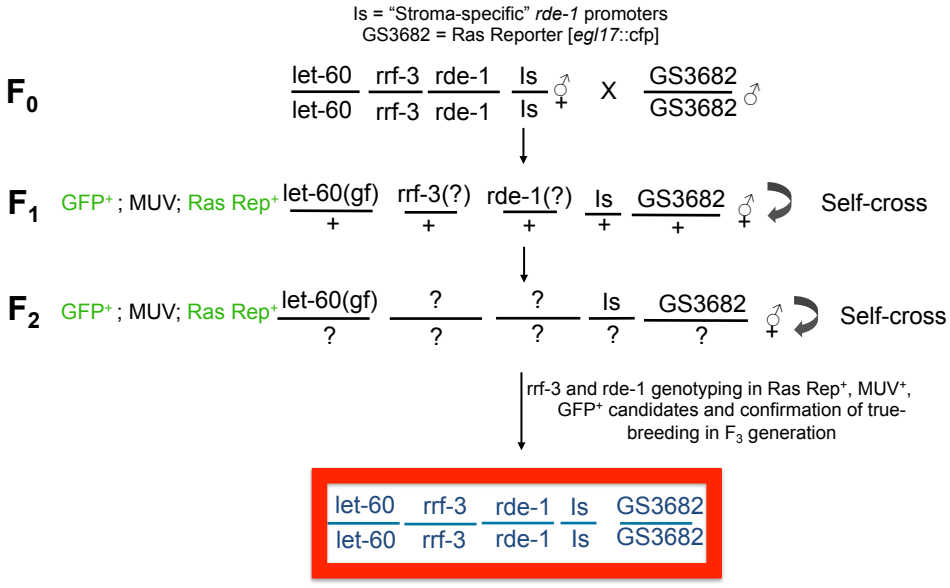

Figure 4. *stroma-rde-1;let-60(lf);rrf-3(lf);Ras Reporter* crossing schematic.

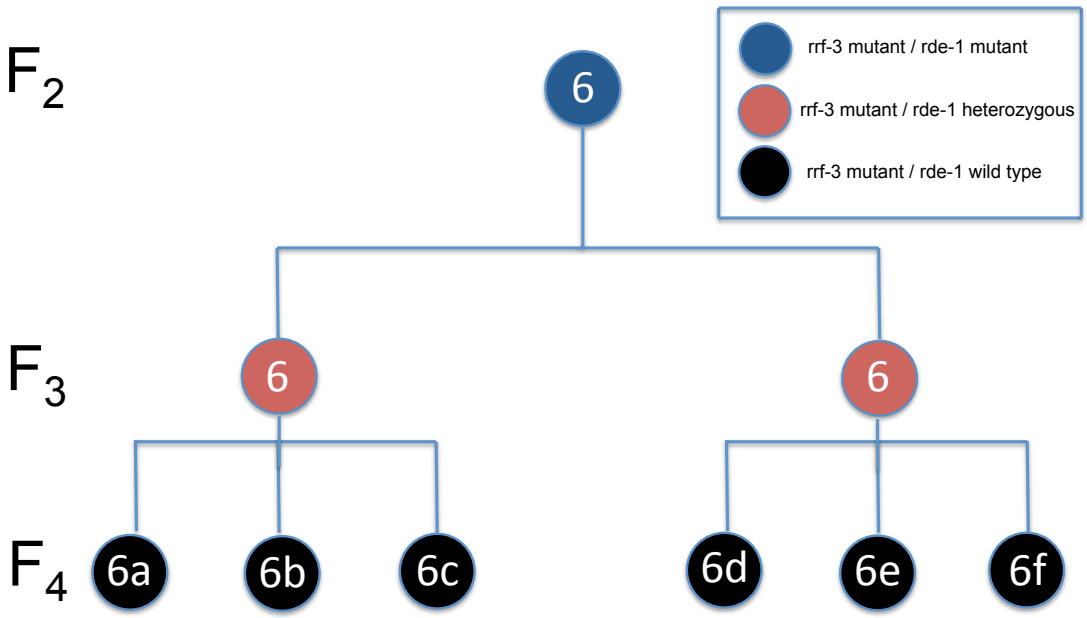

Figure 5. Evolution of *rde-1* genotype in strain 6.

Figure 6. *rrf-3* genotyping of the strain 6. F_4 generation.

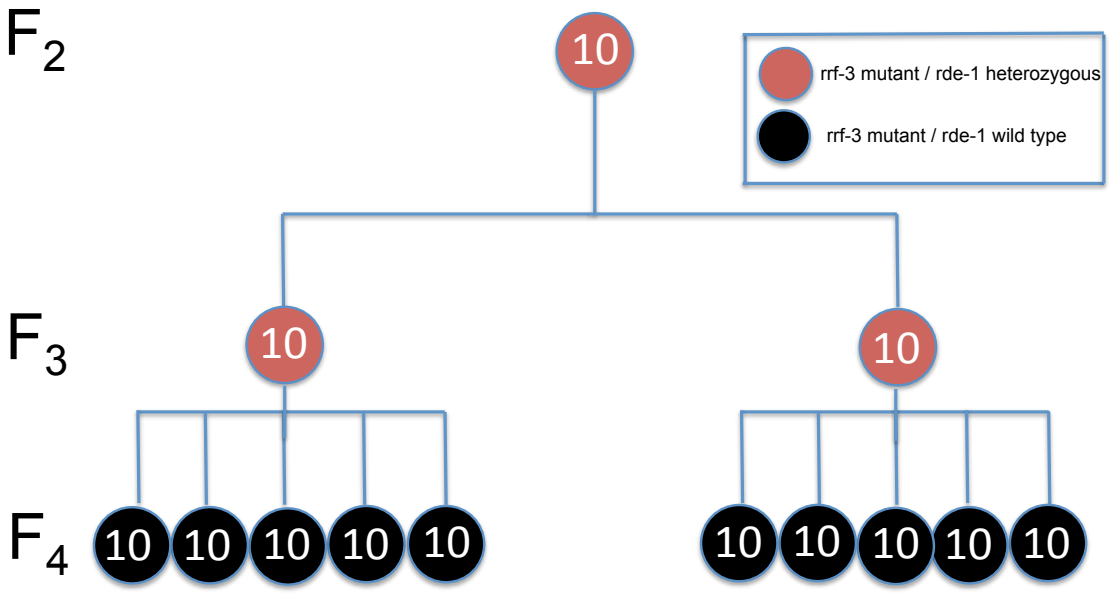

Figure 7. Evolution of *rde-1* genotype in strain 10.

TABLES

Reagent	Volume per Reaction (uL)
DNA	0.5 uL
10x PCR Buffer	2.0 uL
$MgCl_2$	1.6 uL
dNTPs	0.2 uL
Tag	0.2 uL
rrf-3 F- Primer	0.5 uL
rrf-3 -R Primer	0.5 uL
dH_2O	14.5 uL

Reaction Step	Temperature and Time
1	94 °C for 3:00 minutes
2	94 °C for 0:30 minutes
3	55 °C for 0:30 minutes
4	72 °C for 2:00 minutes
5	Go to step 1, 40X
6	72 °C for 3:00 minutes
7	4 °C for ∞

Table 1. *rrf-3* PCR.

Reagent	Volume per Reaction (uL)
DNA	1 uL
10x PCR Buffer	5 uL
$MgCl_2$	4 uL
dNTPs	0.5 uL
Tag	0.5 uL
Primer #1	1 uL
Primer #2	1 uL
dH_2O	37 uL

Reaction Step	Temperature and Time
Step 1	94 °C for 3:00 minutes
Step 2	94 °C for 0:30 minutes
Step 3	55 °C for 0:30 minutes
Step 4	72 °C for 2:00 minutes
Step 5	Go to step 1, 40X
Step 6	72 °C for 3:00 minutes
Step 7	4 °C for ∞

Table 2. *rde-1* PCR.

Chr.	*C. elegans* Gene Name	Mammalian Ortholog	Gene Description
Histone or Histone Regulatory			
II	his-73	HIST2H3D	histone cluster 2, H3d
II	his-43	HIST2H2AB	histone cluster 2, H2ab
IV	his-59	HIST2H3D	histone cluster 2, H3d
IV	his-32	HIST2H3D	histone cluster 2, H3d
IV	his-46	HIST1H4G	histone cluster 1, H4g
V	his-39	HIST2H2BE	histone cluster 2, H2be
V	his-8	HIST2H2BF	histone cluster 2,H2bf
V	his-3	HIST2H2AB	histone cluster 2, H2ab
X	his-71	H3F3B	H3 histone, family 3B (H3.3B)
Membrane Proteins (Transporters and Receptors)			
I	scav-6	SCARB1	scavenger receptor class B, member 1
I	F57B10.5	TMED7	transmembrane emp24 protein transport domain containing 7
I	ncx-4	SLC24A2	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2
II	fgt-1	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3
II	F46B3.9	VLDLR	very low density lipoprotein receptor
X	clec-210	MRC1	mannose receptor, C type 1
X	gyc-11	NPR1	natriuretic peptide receptor 1
X	gpn-1	GPC4	glypican 4
Ribosomal			
I	rpl-24.1	RPL24	ribosomal protein L24
I	C37A2.7	RPLP2	ribosomal protein, large, P2
II	rpl-41	RPL36AL	ribosomal proten L36a-like
II	W01D2.1	RPL37	ribosomal protein L37
IV	lars-1	IARS	isoleucyl-tRNA synthetase
IV	rps-18	RPS18	ribosomal protein S18
Metabolism			
I	F14B4.2	HK2	hexokinase 2
IV	fat-6	SCD	stearoyl-CoA desaturase (delta-9-desaturase)
V	hpo-18	ATP5E	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit
V	atp-4	ATP5J	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6
V	atp-5	ATP5H	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d
V	pyc-1	PC	pyruvate carboxylase
DNA/Cell Cycle Regulation			
I	F25H5.5	CLSPN	claspin
III	W04A8.1	MCPH1	microcephalin 1
III	F54H12.2	RRM2B	ribonucleotide reductase M2 B (TP53 inducible)
Other Functions			
I	C27A12.2	ZNF79	zinc finger protein 79
I	phip-1	PHPT1	phosphohistidine phosphatase 1
II	C26D10.3	PYROXD1	pyridine nucleotide-disulphide oxidoreductase domain 1
II	gst-24	HPGDS	hematopoietic prostaglandin D synthase
IV	mig-28	PRG4	proteoglycan 4
IV	VHA-3	ATP6V0C	ATPase, H+ transporting, lysosomal 16kDa, V0 subunit c
IV	VHA-11	ATP6V1C1	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C1
V	F46B6.5	SRRM5	serine/arginine repetitive matrix 5
V	Y59A8B.19	MUC19	mucin 19, oligomeric
X	lpr-3	TSC22D2	TSC22 domain family, member 2

Table 3. “Candidate genes” with mammalian orthologs identified in genome-wide screen.

Chromosome	*C. elegans* gene	Human Ortholog	*C. elegans expressing MUV*	Total *C. elegans observed*
III	his-59	HIST2H3D	10	44
III	his-32	HIST2H3D	6	41
IV	his-46	HIST1H4G	1	26
IV	lars-1	IARS	5	50
IV	rps-18	RPS18	8	40
IV	fat-6	SCD	9	50
IV	W04A8.1	MCPH1	0	30
IV	F54H12.2	RRM2B	1	31
IV	mig-28	PRG4	16	29
IV	VHA-3	ATP6V0C	13	50
IV	VHA-11	ATP6V1C1	26	50

Table 4. High-magnification validation of candidate genes from chromosomes III and IV.

Cross 1: F_2 Generation			
Strain	**rrf-3**	**rde-1**	**Notes**
1	Mutant	Wild Type	
2	Heterozygous	WT	
3			Deceased
4			Deceased
5	Mutant	Wild Type	
6	Mutant	Mutant	

Table 5. Genotypes of cross 1, F_2 generation.

Strain	Primer	Sequence	rde-1 allelic genotype
6a	rde-1 F	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6a	rde-1 R	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6b	rde-1 F	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTRAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6b	rde-1 R	CCACCTACCACTAGTCAAAGKTAAAAGTGGAGCAAAAGAATACGCTGTRCCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6c	rde-1 F	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6c	rde-1 R	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6d	rde-1 F	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6d	rde-1 R	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6e	rde-1 F	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6e	rde-1 R	CCACCTACCACTAGTCAAAGKTAAAAGTGGAGCAAAAGAATACGCTGTRCCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6f	rde-1 F	CCACCTACCACTAGTCAAAGTTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type
6f	rde-1 R	CCACCTACCACTAGTCAAAGKTAAAAGTGGAGCAAAAGAATACGCTGTACCAATGGAACATCTTGAAGTTCATGAGAAGCCACAAAGATACAA	Wild type

Table 6. *rde-1* genotyping of the strain 6, F_4 generation.

Cross 2: F_2 Generation			
Strain	**rrf-3**	**rde-1**	**Notes**
7			Deceased
8			Deceased
9	Mutant	Wild Type	
10	Mutant	Heterozygous	
11	Heterozygous	Wild Type	
12	Heterozygous	Wild Type	

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